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Encystation of *Giardia lamblia* by High Bile and Alkaline pH and Its Ultrastructural Changes during Encystation

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Abstract *Giardia lamblia*, a human pathogen causing outbreaks of diarrhea, recently became a focus of great concerns in the fields of both medical and environmental microbiology. To develop the experimental tools to study giardiasis, encystation, one of the major processes in its life cycle, was reconstituted by inducing an axenic culture of a flagellated form of *G. lamblia* into a cyst form under high concentration of bile and alkaline pH condition. The successful induction was confirmed by Northern analysis of resulting increased expression of the *CWP1* gene encoding the cyst wall protein 1. An examination of the encystation process with SEM (scanning electron microscopy) and TEM (transmission electron microscopy) revealed that the trophozoite, a flagellate with a bilateral symmetry, was transformed to a cyst form with an oval-shape and defined filamentous wall. The encystation was found to cause a disappearance of the flagella and an invagination of the adhesive disc. An extensive formation of rER (rough endoplasmic reticulum) was observed after 24 h of induction, indicating an active synthesis and export of proteins during this process. The vital staining of the *in vitro*-induced cysts showed that most cysts maintained their viability.

Key words: *Giardia lamblia*, encystation, *CWP1* expression, vital staining

The protozoan parasite *Giardia lamblia* has recently been attracting significant attention of microbiologists for two interesting reasons. According to previous phylogenetic studies inferred from a small subunit rRNA [13, 19] and several protein-coding genes [18, 6], this organism is considered to be a member of the earliest lineage of *Eukarya*. Unlike most eukaryotic organisms, this flagellate lacks a mitochondrion and peroxisome [5]. As a result, some of the metabolic pathways operating in this protozoa are more similar to those in *Bacteria* [2]. Secondly, *G. lamblia* is a worldwide human pathogen that causes diarrheal diseases. Because of recent frequent outbreaks of giardiasis in many countries, the methodology for monitoring and eliminating this protozoa in water supplies is of great concern for public health [3, 4, 7].

The life cycle of *G. lamblia* is composed of two forms, the cyst and the trophozoite. Infection by this protozoa is initiated by the ingestion of the cysts, which then change to being trophozoites by excystation, and, thereafter, the small intestine is colonized by the trophozoites. Before being released into the environment from the host, some trophozoites are induced to transform into cysts, which are rather resistant against harsh environmental conditions. The trophozoite is a pear-shaped flagellate with a bilateral symmetry, adhesive disc, and four pairs of flagella. Within the trophozoite, there are two nuclei with a large central karyosome, two axostyles, and parabasal bodies [12]. In contrast, the cyst has four nuclei and is covered with a well-defined filamentous wall.

In this paper, the induction stage of the trophozoite form into the cyst form, which is a part of the life cycle of *G. lamblia*, was reconstituted. Using the *in vitro*-derived cysts, the molecular and ultrastructural characteristics during the encystation process were carefully examined using Northern hybridization and electron microscopy, respectively. The viability of these cysts was also accessed. Since the reconstitution of encystation *in vitro* produces genetically identical cysts and trophozoites, the study of the molecular mechanism of encystation in *G. lamblia* was possible.

The *G. lamblia* K1 strain isolated from a Korean carrier [15] was grown for 72 h in a normal TYI-S-33 medium (2% casein digest, 1% yeast extract, 1% glucose, 0.2% NaCl, 0.2% L-cysteine, 0.02% ascorbic acid, 0.2% K_2HPO_4, 0.06% KH_2PO_4) [11] containing bovine bile (0.5 mg/ml) at pH 7.1, and then transferred into an encystation medium containing bovine bile (10 mg/ml) at pH 7.8. The induction conditions used in this experiment were adapted as a method of causing encystation, mainly by simulating the physiological conditions under which encystation occurs inside the host [8]. After 24 h-incubation in the encystation medium, the trophozoites were transferred back to the

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normal TYI-S-33 medium to minimize the number of dead cells during the process. Aliquots of portions of parasites were sampled at various times by chilling on ice and collecting them by centrifugation.

To confirm whether the encystation process had been properly induced in this experiment, the expression of the known cyst-specific gene, *CWP1* (for cyst wall protein 1) [13], was monitored by a Northern blot analysis. The full sequence of the *CWP1* gene was obtained from the *G. lamblia* genomic DNA by a PCR using CWP1-F (5'-ACT-AGTATGATGCTCGCTCTCCTTGC-3') and CWP1-R (5'-AAGCTTGAACTGTTCAAAGAGCCG-3') as the forward and reverse primers, respectively. The total RNAs were prepared from the samples collected 6 h, 12 h, and 48 h post-induction as well as from the trophozoites using a TRIzol kit (Gibco BRL, Gaithersburg, U.S.A.) according to the manufacturer's instructions. After separating on a formaldehyde gel and transferring to a nytran filter, the RNAs were then analyzed for the expression of *CWP1* and the control gene. The *TIM* gene coding for triose phosphate isomerase [1] was employed as a control to check the amount of RNA loaded on each lane.

As the trophozoites were incubated in the encystation medium, the expression of *CWP1* gradually increased, showing a maximal expression at 12 h after induction (a 50-fold increase compared to the expression in the trophozoites) (Fig. 1). A similar pattern of induction of the

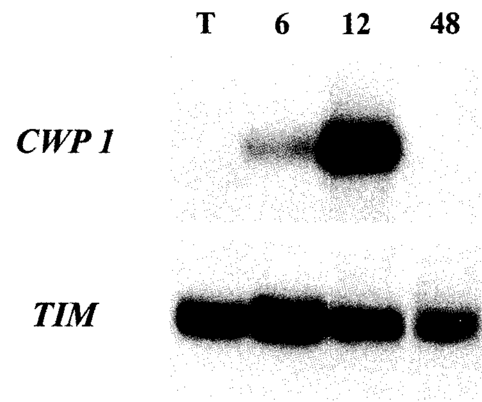

Fig. 1. Expressions of cyst-specific gene, *CWP1* (cyst wall protein 1), and control gene, *TIM* (triose-phosphate isomerase), during *in vitro* encystation.
10 μg of total RNA were used for each sample, harvested during the trophozoite stage (T), 6 h post-induction (6), 12 h post-induction (12), and 48 h post-induction (48).

CWP1 gene during encystation was reported previously [13]. In contrast, the *TIM* gene was expressed constitutively during the encystation process as well as in the trophozoite stage. This reveals that encystation was successfully induced by an alkaline pH and high concentration of bile in this experiment.

To observe the encystation process under electron microscopy (EM), cells were harvested at 0 h (trophozoite

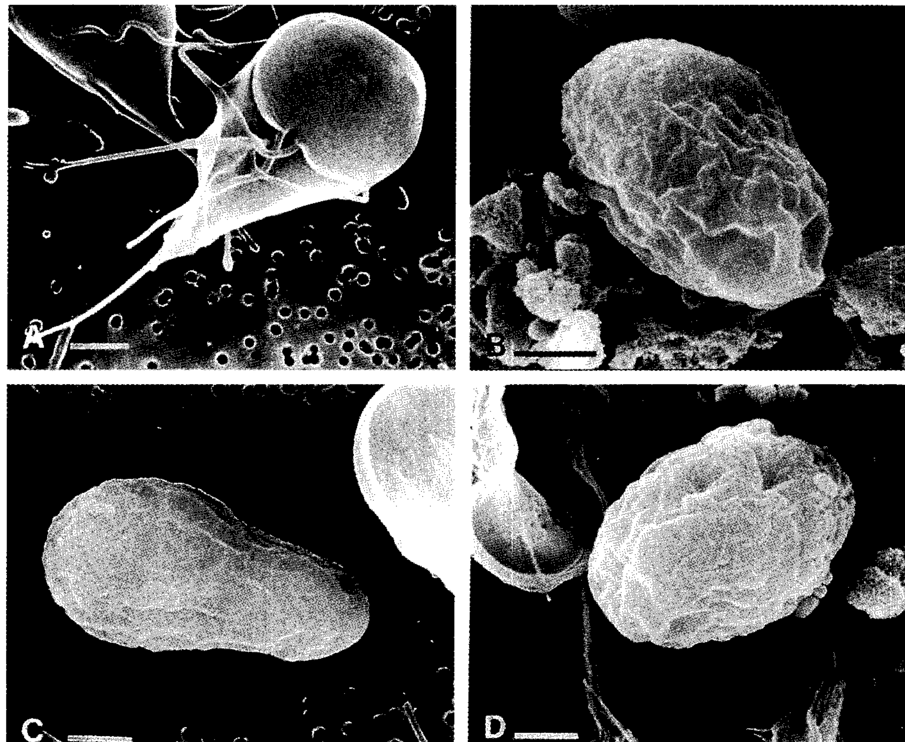

Fig. 2. Scanning electron micrographs of *G. lamblia.*
SEM images of cultivated trophozoite (A), human fecal-originated cyst (B), *in vitro*-derived cysts (48 h-encysting cells) (C and D). Bars, 2 μm.

stage) and 6–48 h after the induction of encystation, and treated as described [9]. Then, the cells were prefixed in a Karnovsky fixative solution (2% glutaraldehyde, 2% paraformaldehyde, 0.5% $CaCl_2$ in a 0.1 M cacodylate buffer (pH 7.4); [10]), followed by washing in 0.1 M cacodylate buffer (pH 7.4) and postfixing by 1.33% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) [16]. Thereafter, the samples were dehydrated in absolute ethanol. For observation by SEM, the dehydrated samples were replaced with isoamyl alcohol and treated with a 300 Å gold coating using an Ion Coater (Eiko IB-3 type). For TEM analysis, the dehydrated samples were replaced by propylene oxides, embedded in an Epon mixture and sectioned. These sections were double-stained with urasyl acetate and lead citrate and observed under a Philips CM-10 TEM. As a control, cysts originated from human fecal samples were included.

In Fig. 2, panels A and B show SEM images of the cultivated K1 trophozoites and human cysts, respectively. The K1 trophozoite shows the presence of flagella and an adhesive disc as in other *Giardia* spp. [12], whereas a cyst from human feces was covered with a weaving thick wall and devoid of flagellum. Aften 48 h of the induction, the trophozoites were found to transform into cysts, as shown

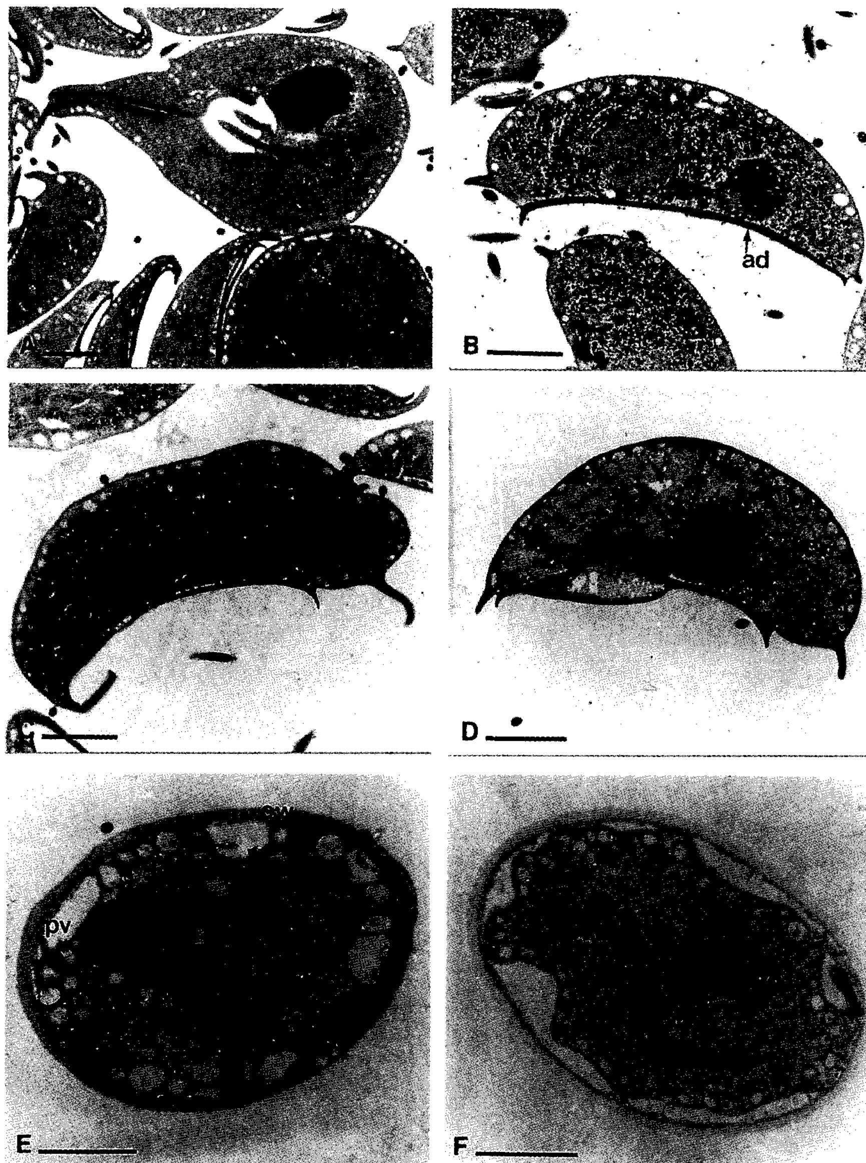

Fig. 3. Cross-sections of trophozoite and encysting *G. lamblia*.
(A) TEM of cultivated trophozoites, (B) TEM of 6 h-encysting cell section, (C) TEM of 12 h-encysting cell section, (D) TEM of 24 h-encysting cell section, (E) TEM of 48 h-encysting cell section, (F) TEM of fecal-originated cyst, showing nuclei (n), adhesive disc (ad), peripheral vesicle (pv), and cyst wall (cw). The arrow in (F) indicates the reminiscent structure of an adhesive disc. Bars, 2 μm.

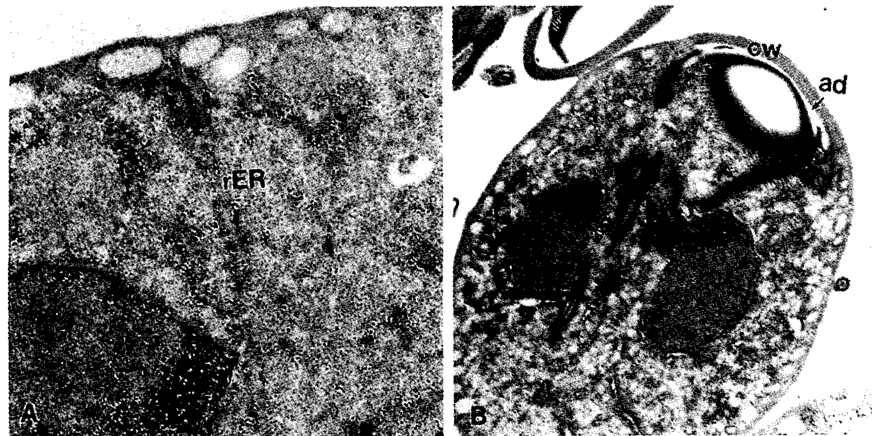

Fig. 4. Higher magnification TEM sections of encysting *G. lamblia*.
(A) 24 h-encysting cell, showing the extensive formation of rER (rough endoplasmic reticulum), magnification x23,000; (B) 6 h-encysting cell, representing the invagination of an adhesive disc (ad) and formation of cyst wall (cw), magnification x12,866.

in Figs. 2C and 2D, which had a similar morphology to the cyst isolated from human feces.

To observe the ultrastructural changes occurring in *G. lamblia* during encystation, encysting samples at various time points were analyzed using TEM. As controls, the TEM for the cultivated trophozoites and fecal cysts are presented in Figs. 3A and 3F, respectively. Figures 3B to 3E are the TEM figures derived from the samples 6, 12, 24, and 48 h post-induction. As the encystation progressed, several structural changes were apparent. First, the peripheral vesicles were fused and became extended. Second, the adhesive disc present in the trophozoite seemed to be invaginated into the interior of the cells. Third, the formation of a distinct filamentous cyst wall was observed outside the cell. Fourth, the appearance of an rER-like structure was noticed in 24 h-encysting cells (Fig. 3D).

Under higher magnification, the formation of rER in the cytoplasm and the invagination of the adhesive disc were evident. At 24 h post-induction, an extensive formation of rER was found, as shown in Fig. 4A. This result suggests that the active production and transport of the cyst proteins occur during this stage. Figure 4B represents the TEM finding at 6 h post-induction and shows the invagination of the adhesive disc. This invagination would appear to produce a reminiscent structure of the disc inside a cyst, as indicated with an arrow in Fig. 3F.

Lastly, using the vital staining method as described by Schupp and Erlandsen [17], it was examined whether these *in vitro*-derived cysts were viable. Briefly, about 10^6 cells at 48 h post-induction were treated with 4 μg of fluorescence diacetate (FDA; Sigma Chemical Co., Saint Lois, U.S.A.) and 3 μg of propidium iodide (PI; Sigma Chemical Co., Saint Lois, U.S.A.) for 5 min. Then, the stained samples were observed under fluorescence microscopy at an excitation wavelength of 546 nm. Due to the differential permeabilities of these two dyes, the live cells will appear fluorescent green by staining with FDA. Since PI is excluded by living cells, only the dead cells will be stained orange-red. Only a minor portion of the cell population appeared orange-red, indicating that most of the cells were alive, evidenced by staining with FDA, and appearing fluorescent green (Fig. 5).

In conclusion, the encystation process of *G. lamblia* was successfully reconstituted. The induction of the cyst-specific gene, *CWP1*, during encystation clearly confirmed that the artificially controlled induction process mimicked the encystation event found in nature. The formation of cysts from trophozoites was observed using EMs. During encystation, a series of ultrastructural changes, including the formation of filamentous cyst walls, disappearance of flagella, invagination of adhesive discs, extensive formation of rER, and fusion of peripheral vesicles, were observed. Combined with the result of the vital staining, these experiments confirmed the formation of viable cysts through *in vitro* encystation. Since *in vitro* encystation produces genetically identical clones of either cysts or trophozoites, this will provide tools for studying the molecular mechanisms

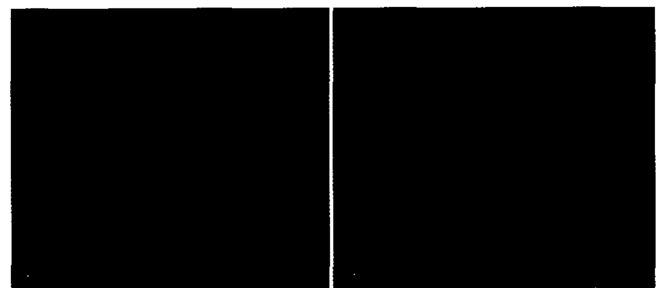

Fig. 5. Vital staining of *in vitro*-derived cysts of *G. lamblia*.
(A) Fluorescence micrograph of *in vitro*-derived cyst of *G. lamblia* stained with PI at an excitation wavelength of 546 nm. (B) Fluorescence micrograph of *in vitro*-derived cyst of *G. lamblia* stained with FDA at an excitation wavelength of 546 nm.

of the encystation of *G. lamblia* within its natural host, humans.

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